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Kajimura et al. J. CELL BIOL. 95, page 217A (Nov. 1982)

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THANKS

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Immunology

Isolation of distinct cDNA clones encoding HLA-DR β chains by use of an expression assay

(Ia antigen/monoclonal antibodies/oocyte injection)

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ABSTRACT cDNA clones encoding different human Ia antigen β chains were isolated by use of a complementation-expression assay in *Xenopus* oocytes. The assay was based on two previous findings. First, oocytes injected with mRNA from a human B-cell line express HLA-DR antigen. The three intracellular DR chains are assembled in oocytes and can be immunoprecipitated with anti-DR monoclonal antibodies. Second, we have isolated cDNA clones encoding DR α and intermediate chains. In order to identify β-chain cDNA clones, mRNA was hybrid-selected with pools of cDNA clones, mixed with mRNA for the α and intermediate chains, and injected into oocytes. We isolated two distinct clones that could select DR β-chain mRNA as demonstrated by assembly of the translation product with DR α chains and immunoprecipitation with DR-specific monoclonal antibodies. One clone is specific for a β chain of the DR locus. The other clone, much weaker in its ability to select DR mRNA, encodes another Ia-like β chain. Full-length cDNA clones corresponding to the DR and Ia-like β chains were isolated and compared. Cross-hybridization was detectable in the coding regions but not in the 3' untranslated regions. Distinct RNAs homologous to the DR and the Ia-like β-chain clones were present in B cells but were undetectable in three T-cell lines.

HLA-DR antigens are highly polymorphic cell surface glycoproteins found primarily on B lymphocytes and macrophages. They are thought to play an important role in the immune response by regulating the interaction among antigen-presenting cells, T cells, and B cells (1). They are also important in the stimulation of the mixed lymphocyte reaction, in allograft rejection, and in their linkage to disease susceptibility (2, 3). Their tissue distribution as well as their biochemical nature suggest that they are the human equivalent of the mouse Ia antigens, encoded in the I region of the mouse H-2 complex (reviewed in ref. 4). HLA-DR antigens are therefore often referred to as human Ia antigens (Fig. 1).

At the cell surface, HLA-DR antigens are made up of two noncovalently linked subunits, the M_r 35,000 α chain and the M_r 29,000 β chain (5). The allelic polymorphism is restricted to the β chain (6–8). Several genes may exist for HLA-DR β chains because structurally distinct β chains have been distinguished with monoclonal antibodies (9). A third subunit—called "invariant" (10), "γ" (11), or "intermediate" (12)—is associated intracellularly with DR antigens. It is thought to be involved in assembly or transport of DR antigens to the cell surface (11, 13).

Serological studies of human Ia antigens have demonstrated that, besides DR, other molecules (e.g., DC-1) exist with as yet undetectable or limited polymorphism. These molecules are coded for by genes in close linkage disequilibrium with the classical polymorphic *DR* locus (14–16). Another locus, termed *SB*, which controls a secondary lymphocyte reaction has been shown to encode Ia antigens (17). The existence of seven different β chains in a *DR*-homozygous cell line has been established biochemically (18). A direct molecular analysis would help understand the relationship between these genes.

We showed previously that *Xenopus* oocytes injected with mRNA from the human B-cell line Raji translate and assemble DR antigens that can be immunoprecipitated with monoclonal antibodies (12). A RNA fraction enriched for the three mRNAs encoding the α, intermediate, and β chains was isolated (12). A cDNA clone encoding the DR α chain, as evidenced by sequence analysis, was isolated (19). Another cDNA clone was isolated which most probably encodes the intermediate chain; its isolation and characterization will be described elsewhere. The availability of the assembly system in oocytes and of cDNA clones encoding α and intermediate chains allowed us to screen for β cDNA clones by a complementation-expression assay using DR-specific monoclonal antibodies. Here we report on the isolation and comparison of several cDNA clones encoding distinct HLA-DR β chains as well as a β chain of another Ia-like locus.

MATERIALS AND METHODS

Preparation of RNA and of cDNA Clones. Cytoplasmic poly(A)$^+$RNA from the human B-cell line Raji (DR 3,w6) was prepared and enriched for HLA-DR mRNA as described (12). Poly(A)$^+$RNA from other sources was prepared by homogenizing frozen cells or tissues in 4 M guanidium thiocyanate buffer as described (20). The homogenate was added to a CsCl gradient and the RNA was pelleted as described for cytoplasmic Raji RNA (12). Production of cDNA clones from the enriched mRNA fraction and the construction of a library of size-selected cDNA clones have been described (19).

Positive Hybrid-Selection of mRNA. Our protocol was modified from the original procedure of Goldberg *et al.* (21). Plasmid DNA was prepared by the standard cleared lysate and ethidium bromide/CsCl density centrifugation procedure, treated with 0.5% diethylpyrocarbonate, passed over a Sepharose 2B column, and then covalently bound to diazobenzyloxymethyl-paper (Schleicher & Schuell) as described (21). On average, 15 μg was bound on a 1-cm^2 filter. Prehybridization was at 37°C for 2–4 hr in 50% (vol/vol) formamide (Merck, recrystallized twice and deionized)/20 mM Pipes, pH 6.4/0.75 M NaCl/2 mM

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Abbreviations: bp, base pair(s); NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate.
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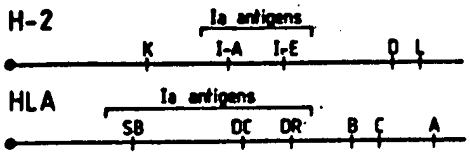

FIG. 1. Loci encoding class I and class II antigens in the mouse (H-2) and the human (HLA) major histocompatibility complex. *H-2K*, *-D*, and *-L* loci in the mouse and *HLA-A*, *-B*, and *-C* loci in the human encode class I antigens which are found on most cells. The *I* region in mouse encodes class II or Ia antigens. The *HLA-D* region in the human is functionally similar to the mouse *I* region. The position of the *DC* and *DR* loci relative to each other is not known. Distances are not to scale. The centromere is indicated by a knob at the left.

EDTA/0.4% $NaDodSO_4$/1% glycine containing *Escherichia coli* tRNA at 0.3 mg/ml and poly(A) at 0.1 mg/ml.

Hybridization with poly(A)$^+$RNA was in the same buffer without glycine, tRNA, and poly(A) at 37°C for 20 hr. Filters were washed three times at 37°C in hybridization buffer, three times at 22°C in 10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.1 M NaCl/0.1% $NaDodSO_4$, and three times at 50°C for 10 min in 10 mM Tris·HCl, pH 7.4/1 mM EDTA. Hybridized mRNA was eluted in two 150-μl portions of 5 mM Tris·HCl, pH 7.4/0.5 mM EDTA containing rabbit tRNA at 6 μg/ml, by heating at 98°C for 75 sec. Eluted RNA was adjusted to 0.3 M sodium acetate (pH 5.0) and precipitated twice with ethanol.

Knowing the sensitivity of the translation assay for DR mRNA injected into *Xenopus* oocytes (12) and the efficiency of the hybrid-selection procedure, we calculated that 1 β cDNA clone in a pool of 50 cDNA clones should be detectable. Eleven 1-cm^2 filters, each loaded with 50 different cDNA clones, and one control filter with pBR322 were incubated with 300 μg of poly(A)$^+$RNA in 200 μl. Under these conditions, the molar mRNA/DNA coding strand ratio was 2:1 for a mRNA representing 0.02% of the mRNA population. In the subsequent rounds of screening, with either 10 clones or 1 clone per filter, recovery of β-chain mRNA was higher because the DNA was in molar excess. Each sample was complemented with mRNA for the α and intermediate chains, which had been selected from 25 μg of poly(A)$^+$RNA under conditions of cDNA excess.

Injection of *Xenopus* oocytes with mRNA and detection of translation products by immunoprecipitation were performed as described (12). Anti-DR rabbit antiserum and monoclonal antibodies D1-12, D4-22, and BT 2.2 have been described (12, 22).

DNA Labeling and Sequence Determination. Restriction fragments were extracted from acrylamide gels and purified over DEAE-cellulose columns (23). Fragments were labeled either internally by the nick-translation procedure (24) or terminally with [α-^{32}P]dNTP (Amersham) and Klenow DNA polymerase I (Boehringer Mannheim). Fragments subcloned in M13 vectors were analyzed by the procedure of Sanger *et al.* (25).

Hybridizations. Dot hybridizations were performed as described (26). Filters were washed several times at 50°C in 4× standard saline citrate (NaCl/Cit; 0.15 M NaCl/0.015 M sodium citrate) before a series of 30-min washes at the indicated stringencies. Gel-transferred RNA bound to diazobenzyloxymethyl-paper was hybridized as described (27) and washed twice for 30 min at each of the following conditions: 50% formamide/5× NaCl/Cit/0.5% $NaDodSO_4$ at 42°C; 50% formamide/2× NaCl/Cit/0.5% $NaDodSO_4$ at 42°C; 5× NaCl/Cit/0.1% sodium pyrophosphate/0.5% $NaDodSO_4$ at 65°C; 1× NaCl/Cit/0.1 $NaDodSO_4$ at 65°C; 0.2× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C; and 0.1× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C. Filters were dehybridized by boiling for 10 min in 2.5 mM Tris·HCl, pH 7.4/0.5 mM EDTA.

RESULTS

Five hundred fifty cDNA clones derived from a Raji mRNA fraction enriched for DR mRNA (12) were grouped in pools of 50. mRNA was selected with each pool by positive hybrid-selection, mixed with mRNA for the α and the intermediate chains, and injected into *Xenopus* oocytes. Each sample was immunoprecipitated with a pool of anti-DR monoclonal antibodies all directed against monomorphic determinants on the DR β chain. In 2 of 11 pools a small amount of DR antigen was synthesized in the injected oocytes. Both positive pools were divided in five pools of 10 clones each and screened again with the complementation-expression assay. One of five pools in each group was again positive (Fig. 2). A final round of screening identified cDNA clones 68-6 and 83-7 as the β-chain clones. The β chain synthesized in oocytes injected with mRNA hybrid-selected with cDNA clone 83-7 was also immunoprecipitated with anti-DR monoclonal antibodies in absence of complementation with α and intermediate chains (Fig. 3). In contrast, cDNA clone 68-6 was much less efficient in selecting DR β-chain mRNA and was not detected in the absence of complementation. This difference between 83-7 and 68-6 was reproduced in three separate experiments.

The size of the cDNA insert was 180 base pairs (bp) for clone 83-7 and 470 bp for clone 68-6. Two long cDNA clones hybridizing with 83-7 at high stringency were isolated from a library of size-selected cDNA clones derived from Raji poly(A)$^+$RNA. Because 83-7 was able to select efficiently, at high stringency, DR β mRNA, as evidenced by assembly with DR α chain in oocytes and immunoprecipitation with anti-DR monoclonal antibodies, we consider that these clones encode DR β chains. They were called DR β-1 and DR β-2 and were found to be similar by restriction enzyme analysis (Fig. 4). Several long cDNA clones, derived from a DR 7,7 homozygous cell line, were also isolated by hybridization with 83-7 (unpublished

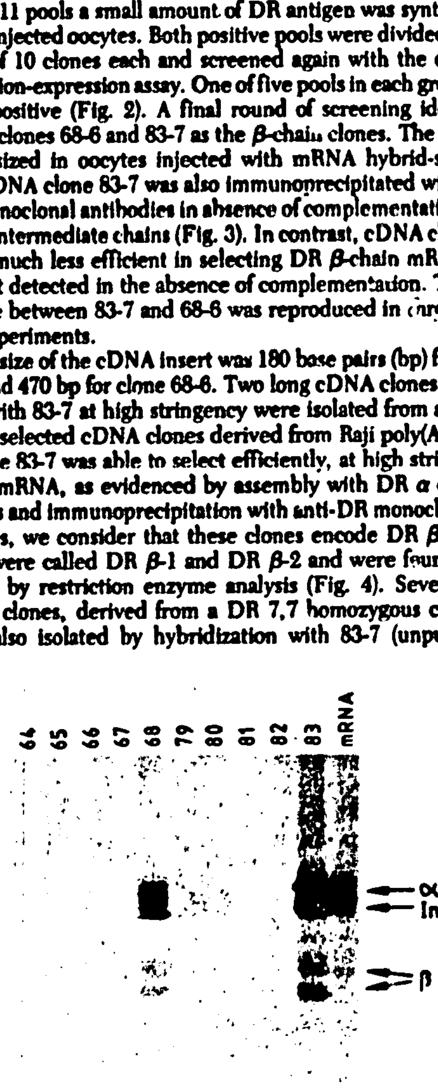

FIG. 2. Identification of cDNA clones encoding β chains by a complementation-expression assay. Each of the two original positive pools of 50 cDNA clones was divided into five pools of 10 clones, numbered 64–68 and 79–83. mRNA selected with these cDNA clones was mixed with mRNA for the α and the intermediate chain. After injection into *Xenopus* oocytes and labeling with [^{35}S]methionine, the translation products were immunoprecipitated with a pool of anti-DR monoclonal antibodies D1-12, D4-22, and BT 2.2 and analyzed in nonreducing 12% $NaDodSO_4$/polyacrylamide gels. Total mRNA was injected into control oocytes (mRNA). The positions of the α, intermediate (In), and β chains are indicated.

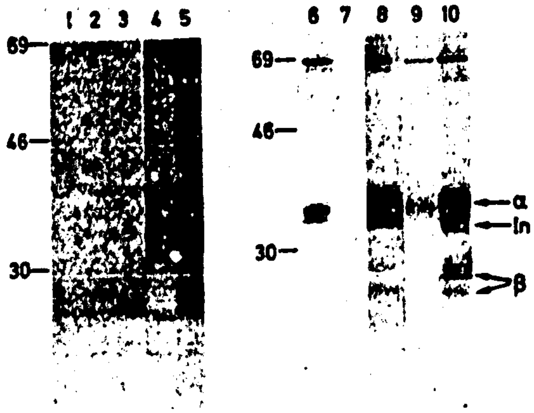

FIG. 3. Identification of two positive β cDNA clones by the complementation-expression assay. *Xenopus* oocytes were injected with mRNA hybrid-selected by use of the following cDNA clones: α and intermediate (lanes 1, 6, and 7); 68-6 (lane 2); 83-7 (lane 3); α, intermediate, and 68-6 (lane 4); α, intermediate, and 83-7 (lane 8). A constant amount of hybrid-selected mRNA was added to each sample. Control oocytes were injected with Raji mRNA (lanes 5 and 9). A lysate of [^{35}S]methionine-labeled Raji cells was included for comparison (lane 10). Translation products were immunoprecipitated with a pool of anti-DR monoclonal antibodies D1-12, D4-22, and BT 2.2 (lanes 1–5 and 7–10) or with a rabbit anti-DR antiserum (lane 6) and analysed in nonreducing 12% NaDodSO$_4$/polyacrylamide gels. The molecular weight of protein markers is given × 10^{-3}. The positions of the α, intermediate (In), and β chains are indicated.

data). The sequence of one of them showed high homology with the amino acid sequence determined by Kratzin *et al.* (18) for a human Ia β chain. A portion of the sequence around the *Hin*fI site first mapped in clone 83-7 is shown in Fig. 5.

A long cDNA clone was isolated from the Raji library by hybridization with 68-6. Because its restriction map was completely different from that of the DR clones, it was called Ia-like β-1 (Fig. 4). The DR and Ia-like clones were compared further by cross-hybridizations at varying stringencies (Fig. 6). DNA sequences from the 3′ untranslated regions did not cross-hybridize. On the other hand, DNA sequences encoding the first

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                HinfI
ACAGTGCAGCGGCGAGTCCATCCTCAGGTGACTGTGTATCCT
 T  V  Q  R  R  V  H  P  Q  V  T  V  Y  P
 -  -  -  -  -  -  -  Q  -  K  -  -  -  -
```

FIG. 5. Nucleotide sequence specifying amino acids 90–103 of a HLA-DR β chain from a DR 7,7 homozygous cell line. The 91-bp *Pst* I fragment of a long cDNA clone was subcloned in mp8 and subjected to sequence analysis in both orientations. The complete sequence of the DR β chain will be published elsewhere. Top line, nucleotide sequence; middle line, deduced amino acid sequence; bottom line, corresponding amino acid sequence of a human Ia β chain from a DR 2,2 homozygous cell line (18) (dashes indicate identity).

domain of β chains did cross-hybridize at an intermediate stringency. Ia-like cDNA clone 68-6, which contains the COOH-terminal portion of the coding sequence, also cross-hybridized with the DR cDNA clone at an intermediate stringency.

Because Ia antigens are found primarily on B cells we tested whether the mRNA corresponding to the DR and the Ia-like β cDNA clones were also B-cell specific (Fig. 7). Both DR β-1 and Ia β-1 hybridized to a RNA species of about 1,300 nucleotides that was expressed in two B-cell lines and in B cells from a patient with chronic lymphocytic leukemia but undetectable in three T-cell lines and in pancreas. A low level of DR β mRNA may be present in liver. In addition to the 1,300-nucleotide-long RNA, each cDNA clone hybridized to an additional and distinct minor RNA species. The DR β-1 clone hybridized to a RNA 1,900 nucleotides long, whereas the Ia-like β-1 clone hybridized to a RNA 1,650 nucleotides long. These longer transcripts are not nuclear RNA precursors because they were present in a cytoplasmic RNA preparation from Raji cells.

DISCUSSION

The strategy for the identification of HLA-DR β-chain cDNA clones was based on two facts. First, *Xenopus* oocytes injected with mRNA from the human B-cell line Raji translate and assemble the α, intermediate, and β DR chains into a complex that can be recognized by anti-DR monoclonal antibodies (12). Second, we have isolated cDNA clones encoding the DR α chain (19). The sequence of the α cDNA corresponds exactly to the known NH_2-terminal sequence of the DR α chain. We have also isolated cDNA clones encoding most likely the human

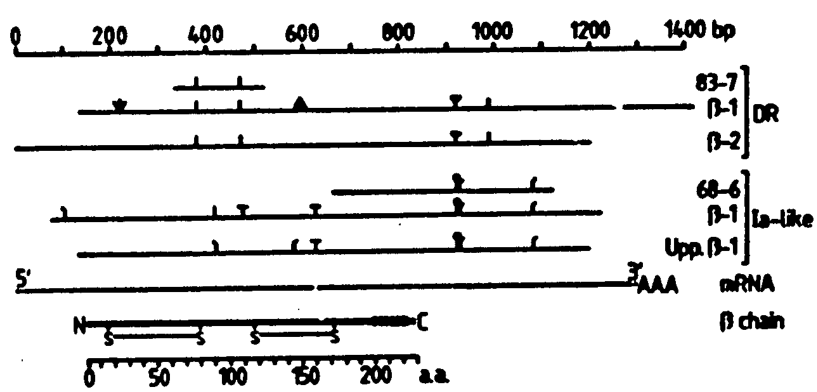

FIG. 4. Maps of the HLA-DR and Ia-like β-chain cDNA clones. Symbols indicate the sites for restriction endonucleases as follows: *Pst* I, ↿; *Hin*dIII, ▼; *Kpn* I, ψ; *Stu* I, ▲; *Eco*RI, ↑; *Pvu* II, ſ; *Sac* I, ↿; *Ava* II, ⊤. Clones 68-6 and 83-7 are the two positive ones identified originally by the complementation-expression assay. DR β-1 and DR β-2 were isolated from a size-selected Raji cDNA library by hybridization with 83-7. Ia-like β-1 was isolated from the same library by hybridization with 68-6. The inserts of all these clones are bound by *Pst* I sites. Upp. β-1 is the clone isolated by Wiman *et al.* (28) in Uppsala and is shown here for comparison. The length of the mRNA has been determined experimentally (see ref. 12 and Fig. 7). The structure of the β chain is derived from the sequence of the 196 NH_2-terminal amino acids determined for a human Ia β chain (18). The alignment of the coding regions for the Ia-like clones is based on the sequence of the Uppsala β-1 clone (29); for the DR clones it is based on the sequence of one of our DR β clones (Fig. 5; unpublished data). a.a., Amino acid.

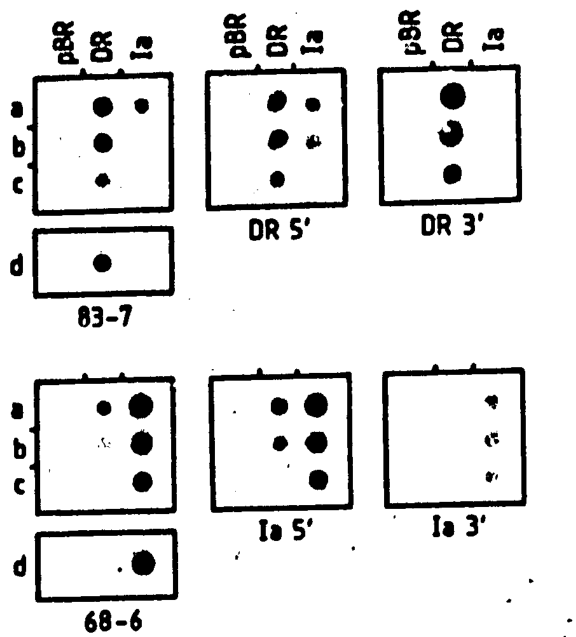

FIG. 6. Cross-hybridizations of DR and Ia-like cDNA clones. Plasmids pBR322 (pBR), DR β-1 (DR), and Ia-like β-1 (Ia) were spotted in vertical rows on nitrocellulose. Filters were hybridized with the following labeled DNA fragments (indicated under each group of spots): insert of clone 83-7 (83-7), insert of clone 68-6 (68-6), *Pst* I fragment C of clone DR β-1 (DR 5'), *Pst* I fragment B of clone DR β-1 (DR 3'), *Pst* I fragment B of clone Ia-like β-1 (Ia 5'), and *Eco*RI/*Pst* I fragment B of clone 68-6 (Ia 3'). The specific activity of the Ia 3' probe was lower than that of the others; therefore it gave weaker signals. The final washes for the different horizontal rows of spots were: a, 50°C, 2× NaCl/Cit, which is 48°C below the t_m of a DNA sequence with 50% G-C base pairs; b, 50°C, 0.1× NaCl/Cit, 24°C below t_m; c, 65°C, 0.05× NaCl/Cit, 5°C below t_m; d, 50°C, 10 mM Tris-HCl, pH 7.4/1 mM EDTA, which corresponds to the final wash in the hybrid-selection procedure.

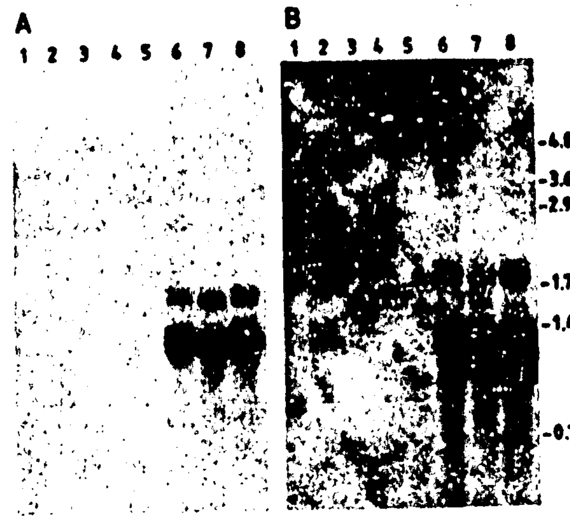

FIG. 7. RNA molecules homologous to DR β-1 and Ia-like β-1 cDNA clones. Poly(A)$^+$RNA (2 μg) was denatured and electrophoresed in a 1.5% agarose gel as described (30) and transferred to diazotized paper (31). The filter was first hybridized with ^{32}P-labeled Ia-like β-1 cDNA (*A*). After autoradiography the filter was dehybridized, checked by autoradiography for the loss of ^{32}P-labeled DNA, and rehybridized with ^{32}P-labeled DR β-1 cDNA (*B*). Total poly(A)$^+$RNA samples were: lane 1, human pancreas; 2, human liver; 3, T-cell line JM; 4, T-cell line CEM; 5, T-cell line Molt 4; 6, B-cell line HHK; 7, B cells isolated from a patient with chronic lymphocytic leukemia; 8, cytoplasmic poly(A)$^+$RNA from the B-cell line Raji. Size markers are indicated in kilobases. Except for human 18S and 28S ribosomal RNA (32), markers were restriction fragments of the plasmid pBR322.

equivalent of the mouse invariant chain (unpublished data), the intermediate chain (12). Because no amino acid sequence is available for these chains, identification of the clones relied on the analysis of translation products. The screening for β cDNA clones was based on the prediction that the translation products of hybrid-selected β mRNA, mixed with mRNA for the α and intermediate chains, would assemble in the oocytes and could be immunoprecipitated with anti-DR monoclonal antibodies.

With this complementation-expression assay a DR β-chain cDNA clone can be identified by two lines of evidence. The first is assembly with DR α chain. Because there is only one α gene hybridizing with α cDNA clones at high stringency (19), the hybrid-selected α mRNA will only encode DR α chain. Heterodimers of DR α chains with other β chains have not been observed *in vivo* (9, 15). Therefore, only DR β chains are expected to assemble with DR α chains. The second line of evidence is based on the use of DR-specific monoclonal antibodies for the immunoprecipitation. These monoclonal antibodies will not detect the translation products of the α- and intermediate-chain mRNAs alone or the α and intermediate chains cotranslated with the mRNAs selected with unrelated cDNA clones (Figs. 2 and 3). Translation products of β mRNA alone were either not detected (with clone 68-6) or detected as a single band comigrating with β chain (with clone 83-7). Only when the three mRNAs were cotranslated did immunoprecipitation of a typical DR complex take place.

One clone, 83-7, was consistently stronger than the other, 68-6, in its ability to select DR β mRNA. We consider therefore that it encodes a DR β chain. Corresponding full-length clones were isolated. The sequence of a cDNA clone derived from the mRNA of a DR 7,7 homozygous cell line shows high homology with the amino acid sequence of a human Ia β chain from a DR 2,2 homozygous line (Fig. 5; ref. 18). This amino acid sequence was determined for the most abundant but undefined β chain (18). We have obtained evidence for at least three DR β-chain genes in the DR 7,7 homozygous line (unpublished data). These DR β-chain genes are homologous to but clearly distinct from other Ia-like β-chain genes. The evidence is based on analysis of cDNA clones and of genomic DNA clones. Therefore, even if the amino acid sequence published by Kratzin *et al.* (18) is of a DR β chain, the differences observed with our cDNA clone do not necessarily reflect allelic polymorphism. For the same reason, the two clones DR β-1 and DR β-2 isolated from the heterozygous line Raji (Fig. 4) are either allelic or nonallelic forms.

A long cDNA clone was also isolated from the Raji cDNA library by hybridization with 68-6 at high stringency; it was called Ia-like β-1. It showed striking similarities in the restriction endonuclease sites with the β-1 clone isolated by Wiman *et al.* (28), also derived from Raji mRNA. Five sites for enzymes recognizing 5 or 6 bp were conserved, indicating a high degree of homology. The amino acid sequence deduced from the sequence of the Uppsala β-1 clone matched only 21 of 34 NH_2-terminal amino acids determined for Raji β chains (28). Therefore, this clone corresponds to a β chain of a minor Ia-like antigen. No evidence was presented for allelic polymorphism of this Ia-like antigen (28). The differences between the Uppsala β-1 clone and our Ia-like β-1 clone can be either allelic or nonallelic.

Structural comparison between DR and Ia-like cDNA clones showed that, although 3′ untranslated regions do not cross-hybridize, coding regions cross-hybridize at an intermediate stringency, indicating about 20% divergence. This observation provides an explanation for the isolation of clone 68-6. Under the same stringency as was used in the mRNA hybrid-selection, clone 68-6 hybridized weakly with the DR β-1 clone. Even though 68-6 may encode not a DR but another Ia-like β chain, it was able to select DR β mRNA with a low efficiency, which was then identified in the complementation-expression assay for DR antigen.

The *in vivo* expression of the DR and the Ia-like β chains was analyzed at the RNA level. Both β-chain mRNAs were B-cell specific. Three different T-cell lines were negative, as was pancreas. In addition to the major RNA species, each cDNA clone hybridized to distinct longer RNA molecules. It is not clear whether these longer RNA molecules represent different transcripts of the same gene or transcripts from different genes. The DR β-1 cDNA clone has a long 3′ sequence and may be derived from a longer transcript.

In conclusion, we have isolated full-length cDNA clones for two distinct β chains of human Ia antigens. One encodes a DR β chain; the other corresponds to another locus, which can be either *DC*, *SB*, or an as yet unidentified locus. The DR β-chain cDNA clone and the other β-chain cDNA clone show moderate sequence conservation in the coding regions but complete divergence in the 3′ untranslated regions.

Note Added in Proof. The Ia-like β clone has a strong homology to the mouse I-A β chain gene (33) and thus may correspond to HLA-DC. Extensive polymorphism was detected with the DR and the Ia-like β clones in human DNA (34).

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